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¹⁸F-radiolabeled analogs of exendin-4 for PET imaging of GLP-1 in insulinoma

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Abstract

Purpose—Glucagon-like peptide type 1 (GLP-1) is an incretin peptide that augments glucose-stimulated insulin release following oral consumption of nutrients. Its message is transmitted via a G protein-coupled receptor called GLP-1R, which is colocalized with pancreatic β -cells. The GLP-1 system is responsible for enhancing insulin release, inhibiting glucagon production,

inhibiting hepatic gluconeogenesis, inhibiting gastric mobility, and suppression of appetite. The abundance of GLP-1R in pancreatic β -cells in insulinoma, a cancer of the pancreas, and the activity of GLP-1 in the cardiovascular system have made GLP-1R a target for molecular imaging.

Methods—We prepared ^{18}F radioligands for GLP-1R by the reaction of [^{18}F]FBEM, a maleimide prosthetic group, with [Cys⁰] and [Cys⁴⁰] analogs of exendin-4. The binding affinity, cellular uptake and internalization, in vitro stability, and uptake and specificity of uptake of the resulting compounds were determined in an INS-1 xenograft model in nude mice.

Results—The [^{18}F]FBEM-[Cys^x]-exendin-4 analogs were obtained in good yield ($34.3\pm3.4\%$, $n=11$), based on the starting compound [^{18}F]FBEM, and had a specific activity of 45.51 ± 16.28 GBq/ μmol (1.23 ± 0.44 Ci/ μmol , $n=7$) at the end of synthesis. The C-terminal isomer, [^{18}F]FBEM-[Cys⁴⁰]-exendin-4, had higher affinity for INS-1 tumor cells (IC_{50} 1.11 ± 0.057 nM) and higher tumor uptake (25.25 ± 3.39 %ID/g at 1 h) than the N-terminal isomer, [^{18}F]FBEM-[Cys⁰]-exendin-4 (IC_{50} 2.99 ± 0.06 nM, uptake 7.20 ± 1.26 %ID/g at 1 h). Uptake of both isomers into INS-1 tumor, pancreas, stomach, and lung could be blocked by preinjection of nonradiolabeled [Cys^x]-exendin-4 ($p<0.05$).

Conclusion—[^{18}F]FBEM-[Cys⁴⁰]-exendin-4 and [^{18}F]FBEM-[Cys⁰]-exendin-4 have high affinity for GLP-1R and display similar in vitro cell internalization. The higher uptake into INS-1 xenograft tumors exhibited by [^{18}F]FBEM-[Cys⁴⁰]-exendin-4 suggests that this compound would be the better tracer for imaging GLP-1R.

Keywords

Exendin-4; GLP-1R; Insulinoma; ^{18}F ; PET

Introduction

The glucagon-like peptide type-1 (GLP-1) is released in the gut following ingestion of nutrients. The peptide is responsible for the stimulation of insulin release from the β -cells of the pancreas to maintain glucose homeostasis. GLP-1 is an incretin that augments the glucose-dependent secretion of insulin, inhibits glucagon production, inhibits hepatic gluconeogenesis, inhibits gastric mobility, and suppresses appetite [1, 2]. Because of this stimulation of insulin release, it is not surprising to find that the target receptor for GLP-1 (GLP-1R) is highly expressed in pancreatic β -cells [3]. GLP-1 must, of necessity, be controlled in its actions to prevent development of hypoglycemia as the blood glucose concentration is reduced by the actions of insulin. Consequently, the native peptide is rapidly degraded by dipeptidyl-peptidase-IV via cleavage of two N-terminal residues, which abolishes the agonistic effects at GLP-1R [4].

Diabetes is a serious and increasingly common medical condition in which the blood glucose levels are abnormal. Type 1 diabetes is characterized by lack of insulin production in the pancreas, and type 2 diabetes is characterized by high blood glucose concentrations that are often resistant to insulin concentration. Type 2 diabetes is accompanied by a loss of the efficiency of the GLP-1R/GLP-1 system. Secretion of GLP-1 is reduced and the potency of GLP-1 at its receptor is also reduced [1]. GLP-1 agonists and inhibitors of dipeptidyl-peptidase-IV, which prolong the biological half-life of GLP-1, are used in the treatment of type 2 diabetes. Because of the high prevalence of diabetes in the human population, there is keen interest in developing radioactive imaging agents that can be applied to study of the causes and treatment of this disease [5, 6].

GLP-1R has been shown to be expressed in high density and high incidence in certain types of cancers derived from endocrine, neuroendocrine, and embryonic origins [7]. Benign

insulinoma is a neuroendocrine cancer of pancreatic β -cells that displays high levels of both GLP-1R and somatostatin receptors [7]. It is a rare form of cancer, and its lesions are difficult to detect by standard clinical imaging methods due to their small size and the anatomical location in the pancreas. The INS-1 (832/13) cell line is a rat insulinoma cell line with positive GLP-1R expression [8]. The fact that GLP-1R is one of the target receptors for diabetes imaging makes the INS-1 tumor an excellent tissue model to study novel ligands.

Exendin-4 is a subcutaneously administered peptide drug that is used in the treatment of type 2 diabetes. Originally discovered in the saliva of a lizard [9], exendin-4 was the first of a class of incretin mimetics that showed potent glucoregulatory activity. Research groups have explored GLP-1 imaging with analogs of exendin that have been designed to complex radiometals such as ^{111}In and $^{99\text{m}}\text{Tc}$ for single-photon emission computed tomography (SPECT) and ^{68}Ga for PET [10–12]. ^{123}I -GLP-1(7–36) amide and ^{123}I -exendin-3 have been studied in a subcutaneous insulinoma model [13]. Images of insulinoma xenografts were obtained with these ^{123}I -labeled peptides, but both compounds exhibited rapid washout. Wild et al. [10] prepared an ^{111}In -DTPA-exendin-4 analog, and successfully imaged small insulinomas in the Rip1Tag2 mouse model. This radiotracer demonstrated therapeutic potential at higher doses [14]. This same compound has been utilized clinically to image insulinomas using SPECT [15, 16]. ^{68}Ga - and $^{99\text{m}}\text{Tc}$ -labeled exendin-4 analogs have also been evaluated [11]. One application for these imaging agents is the study of pancreatic β -cell mass during both the course of diabetes development and monitoring of islet transplantation. The GLP-1 receptor radioligand, $[\text{Lys}^{40}(\text{Ahx-DTPA-}^{111}\text{In})\text{NH}_2]\text{exendin-4}$, provided imaging of functional β -cells 1 year following islet transplantation into the forearm of a human patient [17].

We have previously prepared ^{18}F FBEM-EM3106B as a PET imaging agent and evaluated it in the INS-1 xenograft model [18]. EM-3106B was developed to enhance the biological half-life of this class of molecules by inclusion of two cyclic lactam bridges in the structure of the peptide [19]. We extended our previous work by preparing ^{18}F -labeled analogs of exendin-4. Exendin-4, which contains no cysteine residue in the native structure, was modified with either a C-terminal or N-terminal cysteine to allow site-specific labeling with ^{18}F FBEM, a maleimide-selective prosthetic reagent. The cell uptake, biodistribution, and imaging properties of these ^{18}F FBEM-conjugated analogs were studied using the INS-1 tumor cell and xenograft models.

Materials and methods

Preparation and analysis of FBEM-exendin-4 analogs

$[\text{Cys}^0]$ -exendin-4 and $[\text{Cys}^{40}]$ -exendin-4 (FW 4289.69) were prepared by solid-phase peptide synthesis (CS Bio, Menlo Park, CA, USA). The exendin-4 sequence for the $[\text{Cys}^{40}]$ analog is $[\text{His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gly-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-Cys-NH}_2]$ and for the $[\text{Cys}^0]$ analog is $[\text{Cys-His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gly-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH}_2]$. N -[2-(4-Fluorobenzamido)ethyl]maleimide (FBEM) was prepared as previously described [20]. ^{18}F FBEM was synthesized using an automated system as previously described [21]. For semipreparative HPLC, a Vydac C-18 protein column (9.4×250 mm) and a gradient elution profile were used with 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 0.1% TFA in CH_3CN (solvent B). For analytical HPLC, a Vydac C-18 protein column (4.6×250 mm) was used with a gradient elution profile using water, CH_3CN , 1 mM EDTA Na_2 , and 1% TFA in water (solvent C). For HPLC mass spectrometry, a Waters QTOF coupled to a Waters Acquity UPLC and gradient profile were used with formic acid buffers.

General procedure for synthesis of nonradioactive FBEM-exendin-4 analogs—

[Cys^x]-Exendin-4 (2–5 mg) was dissolved in 300 μ l degassed phosphate-buffered saline (PBS) and treated with FBEM (5 equivalents) in 100 μ l CH₃CN. The solution was incubated for 1 h and then injected onto the semipreparative HPLC column. The elution profile was isocratic at 25% solvent B for 5 min, then a gradient to 55% solvent B over 20 min, and finally to 90% B over the next 5 min. The major peak at about 17 min was collected and lyophilized.

FBEM-[Cys⁰]-exendin-4: Analytical HPLC t_R =18.3 min. HPLC-MS 1,517.8 [M+3H]³⁺; deconvolves to 4,551. Calculated exact mass: C₂₀₀H₂₉₇FN₅₃O₆₄S₂ (4,550.887). HPLC-MS-MS m/z: 503 (H-FBEM-Cys-His)⁺; 396 (Pro-Pro-Pro-Ser-NH₃)⁺; 299 (Pro-Pro-Ser-NH₃)⁺; 202 (Pro-Ser-NH₃)⁺.

FBEM-[Cys⁴⁰]-exendin-4: Analytical HPLC t_R =18.3 min. HPLC-MS 1,517.8 [M+3H]³⁺; deconvolves to 4,551. Calculated exact mass: C₂₀₀H₂₉₇FN₅₃O₆₄S₂ (4,550.887). HPLC-MS-MS m/z: 761 (Pro-Pro-Pro-Ser-Cys-FBEM-NH₃)⁺; 664 (Pro-Pro-Ser-Cys-FBEM-NH₃)⁺; 567 (Pro-Ser-Cys-FBEM-NH₃)⁺.

General procedure for synthesis of oxidation product of FBEM-[Cys^x]-

exendin-4—FBEM was conjugated with [Cys^x]-exendin-4 as described above. After a 30-min reaction time, the reaction was treated with H₂O₂ in CH₃COOH for 10 min. The entire reaction mixture was then separated by semipreparative HPLC using the same conditions as described above. The major peak was collected as the oxidation product.

FBEM-[Cys⁰]-exendin-4 oxide: Analytical HPLC t_R =15.9 min. HPLC-MS 1,523.5 [M+3H]³⁺; 1,142.9 [M+4H]⁴⁺ deconvolves to 4,568. Calculated exact mass: C₂₀₀H₂₉₇FN₅₃O₆₅S₂ (4,566.886).

FBEM-[Cys⁴⁰]-exendin-4 oxide: Analytical HPLC t_R =15.9 min. HPLC-MS 1,523.5 [M+3H]³⁺; 1,142.9 [M+4H]⁴⁺ deconvolves to 4,568. Calculated exact mass: C₂₀₀H₂₉₇FN₅₃O₆₅S₂ (4,566.886).

Radiochemical synthesis of [¹⁸F]FBEM-[Cys^x]-exendin-4—[¹⁸F]FBEM was prepared as previously described and obtained as a solution in CH₂Cl₂ [18, 21]. The yield of [¹⁸F]FBEM prepared for these studies was 18.8 $\pm$ 3.1% (uncorrected). The desired amount of [¹⁸F]FBEM (range 388.5–984.2 MBq) was evaporated to near dryness. A small amount of water remained. Ethanol (10 μ l) was added to the tube and the tube was vortexed. [Cys^x]-Exendin-4 (100–200 μ g) in 100 μ l 0.1% sodium ascorbate in PBS (ultrasonically degassed) was added, and the solution was vortexed followed by incubation for 30 min. Aqueous TFA (0.1% TFA, 100 μ l) was added and the solution injected onto a semipreparative HPLC system. The major radioactivity peak was collected as product. The elution time of the radioactive product was about 17.9 min. The radioactive oxidation product eluted at about 15.1 min and unreacted [Cys⁰]- or [Cys⁴⁰]-exendin-4 eluted at about 17.1 min. The disulfide dimer of [Cys⁰]- or [Cys⁴⁰]-exendin-4 eluted after the desired FBEM conjugate at about 18.7 min. The fraction containing [¹⁸F]FBEM-[Cys⁰]- or [¹⁸F]FBEM-[Cys⁴⁰]-exendin-4 was diluted to 20 ml with water and the solution passed through a C-18 BondElut cartridge (100 mg). The product that remained on the cartridge was eluted with 1 ml of 10 mM HCl in ethanol. The ethanol was concentrated on a rotary evaporator to about 100–200 μ l. A portion of this remaining volume was used for determination of radiochemical purity and specific activity. The remaining volume was diluted in PBS for in vitro and in vivo studies.

Quality control—For analytical HPLC the column described in general methods was used. The gradient used was 65% water, 25% CH₃CN, 10% solvent C for 5 min, then a linear gradient to 35% water, 55% CH₃CN, 10% solvent C by 25 min, and finally, a linear gradient to 90% CH₃CN, 10% solvent C at 30 min. FBEM-[Cys⁰]- or FBEM-[Cys⁴⁰]-exendin-4 eluted at about 18.3 min and the corresponding oxidation product eluted at about 15.9 min. The included mass was estimated based on a standard curve of authentic standard at wavelengths of 210 nm, 230 nm, 250 nm, and 280 nm. An average mass value calculation from the four wavelengths was used to estimate the specific activity of the sample.

Plasma and serum stability—[¹⁸F]FBEM-[Cys⁴⁰]-exendin-4 was mixed with 200 μl of human plasma or mouse serum and a 50-μl aliquot was removed at 0 min. The remainder was incubated at 37°C and additional aliquots of 50 μl were removed at 30 and 60 min. The aliquots were mixed with an equal volume of CH₃CN, the layers were assayed to determine extraction efficiency, and a portion of the supernatant was subjected to radioHPLC analysis using an on-line radioactivity detector or collection of 1-min fractions and counting in a γ-counter (Wallach Wizard, PerkinElmer).

Cell culture and animal model

All animal studies were conducted in accordance with the principles and procedures outlined in the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the Clinical Center, NIH. The rat insulinoma INS-1-derived 832/13 cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Invitrogen), and in a humidified atmosphere containing 5% CO₂ at 37°C. The MDA-MB-435 cell line [22] was purchased from the American Type Culture Collection (ATCC) and grown in Leibovitz's L-15 medium supplemented with 10% (v/v) fetal bovine serum under an atmosphere of 100% air at 37°C. The INS-1 and MDA-MB-435 xenograft tumor models were developed in 5- to 6-week old female athymic nude mice (Harlan Laboratories) by injection of 5×10⁶ cells into the left or right shoulder. Tumor growth was monitored by measuring perpendicular axes of the tumor using a caliper. The tumor volume was estimated from the formula: tumor volume= $a \times (b^2)/2$, where a and b are the tumor length and width, respectively, in millimeters. The mice underwent a small-animal PET scan when the tumor volume reached 100–300 mm³ (3–4 weeks after inoculation).

Cell binding assay

In vitro GLP-1R binding affinity and specificity of FBEM-[Cys⁰]- or FBEM-[Cys⁴⁰]-exendin-4, FBEM-[Cys⁴⁰]-exendin-4 oxide, and GLP-1 were assessed via a competitive cell binding assay using [¹²⁵I]-GLP-1(7–36) as the GLP-1R-specific radioligand. Experiments were performed on triplicate samples of rat INS-1 cells. The best-fit 50% inhibitory concentrations (IC₅₀) for the INS-1 cells were calculated by fitting the data with nonlinear regression using GraphPad Prism (GraphPad Software).

Cell uptake, internalization, and efflux studies

For cell uptake, INS-1 cells were seeded into 24-well plates at a density of 1×10⁵ cells per well and incubated with 18.5 kBq (0.5 μCi/5 ng at the time of the experiment) per well of [¹⁸F]FBEM-[Cys⁴⁰]- or [¹⁸F]FBEM-[Cys⁰]-exendin-4 at 37°C for 15, 30, 60, and 120 min. The cells were then washed three times with chilled PBS and lysed with 200 μl 0.1 M NaOH. Nonspecific binding was determined by comparing the cell uptake with and without an excess of 0.1 M [Cys⁴⁰]- or [Cys⁰]-exendin-4. For the determination of internalization, surface-bound radiotracer was removed by washing the cells three times with an acid buffer (50 mM glycine and 0.1 M NaCl, pH 2.8). The remaining cell activity constituted

internalized tracer. For efflux studies, about 18.5 kBq (0.5 μ Ci) per well of [18 F]FBEM-[Cys⁰]- or [18 F]FBEM-[Cys⁴⁰]-exendin-4 were first incubated with INS-1 cells in 24-well plates for 2 h at 37°C. The cells were washed three times with chilled PBS and allowed to stand with fresh buffer at 37°C. At various time points, the medium was removed and the cells washed three times with chilled PBS. The cells were then lysed with 200 μ l 0.1 M NaOH. The cell lysate was collected and the remaining radioactivity was measured in the γ -counter. The cell uptake, internalization and efflux were expressed as the percentage of the added dose (%AD) after decay correction. All experiments were performed twice with triplicate wells.

MicroPET imaging

PET scans and image analysis were performed using an Inveon microPET scanner (Siemens Medical Solutions). [18 F]FBEM-[Cys⁴⁰]- or [18 F]FBEM-[Cys⁰]-exendin-4 (3.44 $\pm$ 0.26 MBq, about 100 μ Ci, containing 0.5 to 1 μ g estimated from average specific activity with allowance for decay to time of injection) was injected via a tail vein under isoflurane anesthesia. Five-minute static PET images were acquired at 1 h and 2 h after injection (six animals per group). For the GLP-1R blocking experiment, 200 μ g [Cys⁰]- or [Cys⁴⁰]-exendin-4 (corresponding to the radiolabeled isomer) was coinjected with 3.7 MBq (100 μ Ci) of [18 F]FBEM-[Cys⁴⁰]- or [18 F]FBEM-[Cys⁰]-exendin-4 into INS-1 tumor-bearing mice and 5-min static PET images were acquired at the 1-h time point (four animals). The images were reconstructed using a two-dimensional ordered-subsets expectation maximization (2-D OSEM) algorithm without correction for attenuation or scattering. For each scan, regions of interest (ROIs) were drawn over the tumor and major organs using vendor software (ASI Pro 5.2.4.0) on decay-corrected whole-body coronal images. The radioactivity concentrations (accumulation) within the tumors, muscle, liver, and kidneys were obtained from mean pixel values within the multiple ROI volume and then converted to megabecquerels per milliliter per minute using the calibration factor determined for the Inveon PET system. These values were then divided by the administered activity to obtain (assuming a tissue density of 1 g/ml) an image ROI-derived percent injected dose per gram (%ID/g).

Ex vivo biodistribution

Immediately after PET imaging, the tumor-bearing mice were killed and dissected. Blood, tumor, major organs, and tissues were collected and wet-weighed. The radioactivity in the wet whole tissue was measured with a γ -counter. The results were expressed as percentages of the injected dose per gram of tissue (%ID/g) for a group of six animals. For each mouse, the radioactivity of the tissue samples was calibrated against a known aliquot of the injected radio-tracer. Values are expressed as means $\pm$ SD. For the blocking study, tumor xenografted mice were pretreated with 200 μ g [Cys⁴⁰]- or [Cys⁰]-exendin-4 10 min prior to injection of the corresponding [18 F]FBEM-[Cys^x]-exendin-4.

Western blot analysis of GLP-1R

INS-1 and MDA-MB-435 tumor samples (100–500 mg) frozen in liquid nitrogen were homogenized and suspended in RIPA buffer solution (150 mM NaCl, 1.0% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, and 1 mM orthovanadate). Soluble protein fractions (30 μ g) were separated on 12% polyacrylamide gels using SDS-PAGE and transferred to nitrocellulose membranes [23], which were blocked for 1 h at room temperature with TBS containing 5% bovine serum albumin. The blots were washed with TBS containing 0.1% Tween 20 and then incubated with rabbit anti-rat polyclonal GLP-1R antibody (1:500, Abcam). In all determinations, monoclonal antibody for rat β -actin (1:2,000, Abcam) was used as an internal control. After

extensive washing, the antigen–antibody complexes were detected using horseradish peroxidase-labeled donkey anti-rabbit IgG and a SuperSignal West Pico chemiluminescence kit detection system (Pierce, Rockford, IL).

Statistical analysis

Quantitative data are expressed as means $\pm$ SD. Means were compared using one-way analysis of variance and Student's *t*-test. A *p* value of <0.05 was considered statistically significant.

Results

Syntheses

The chemical and radiochemical syntheses provided adequate amounts of the desired chemical entities. The identity of the two peptides could be distinguished on the basis of MS–MS data that showed fragments derived from the C-terminal portion of the peptide. Thus FBEM-[Cys⁴⁰]-exendin-4 showed the addition of FBEM in the four C-terminal fragments observed, while FBEM-[Cys⁰]-exendin-4 did not. Both products exhibited a tendency to form a monooxidation product that we hypothesize resulted from the conversion of the methionine residue into its corresponding sulfoxide.

The radiochemical syntheses provided yields for [¹⁸F] FBEM-[Cys⁰]-exendin-4 and [¹⁸F]FBEM-[Cys⁴⁰]-exendin-4 of 34.3 $\pm$ 3.4% (*n*=11) based on the starting [¹⁸F]FBEM not corrected for decay. There was no difference in yield between the two isomers. Since the yield of [¹⁸F]FBEM for this study averaged 18.8%, the uncorrected yield of the [¹⁸F]FBEM-[Cys^x]-exendin-4 analogs based on the starting [¹⁸F]fluoride was about 6.4%. The average specific activity of all preparations was 45.51 $\pm$ 16.28 GBq/ μ mol (1.23 $\pm$ 0.44 Ci/ μ mol; *n*=7) at the time of measurement at the end of the radiochemical synthesis. With these specific activities, a typical imaging dose of 3.7 MBq (100 μ Ci) would contain about 81 pmol (0.37 μ g) mass at the end of synthesis. Radiochemical purity was $>96\%$ at the time of preparation. A minor impurity peak identified as an oxidation product (see text below) was observed as contaminant. This minor oxidation impurity increased slightly over time upon standing.

In vitro affinity

In our initial radiochemical syntheses, oxidation was a significant issue. Because of this issue, we evaluated the affinity of the oxidation product compared to unoxidized product in a cell binding assay vs. [¹²⁵I]GLP-1(7–36). The measured affinities were 1.88 $\pm$ 0.075 nM for FBEM-[Cys⁴⁰]-exendin-4 oxide and 1.22 $\pm$ 0.049 nM for FBEM-[Cys⁴⁰]-exendin-4 (Fig. 1). In a separate assay the two isomers were compared. The IC₅₀ values were 1.11 $\pm$ 0.057 nM, 2.99 $\pm$ 0.0056 nM, and 5.33 $\pm$ 0.053 nM for FBEM-[Cys⁴⁰]-exendin-4, FBEM-[Cys⁰]-exendin-4, and GLP-1, respectively.

Human plasma and mouse serum stability

Stability in biological fluids was examined for FBEM-[Cys⁴⁰]-exendin-4. Extraction efficiencies from human plasma were 91%, 72%, and 68% at 0, 30, and 60 min, respectively. In human plasma, only a minor amount of radiolabeled oxidation product was observed in the samples. Parent radiolabeled peptide comprised 92%, 89%, and 88% of the radioactivity at 0, 30, and 60 min, respectively. Mouse serum extraction efficiencies were 87%, 85%, and 82% at 0, 30, and 60 min, respectively. Only a trace amount of a very polar radioactive component was observed that increased slightly over time in the serum incubation. At 60 min, 88% of the radioactivity was collected in the parent product peak. Radioactivity recovery based on gamma counting of collected fractions was 94% for the

mouse sample at 60 min, 98% for the human sample at 0 min, and 90% for the human sample at 60 min (Fig. 2).

Cell uptake and clearance

Cellular uptake in vitro was modest with the majority of the radioactivity internalized. FBEM-[Cys⁴⁰]-exendin-4 showed slightly higher cellular uptake than the [Cys⁰] isomer. The proportion internalized was not different between the two isomers. Cellular uptake was inhibited with a blocking concentration. The efflux appeared biphasic with an early rapid phase, probably reflecting loss of surface binding, followed by a slow phase representing clearance of the intracellular pool (Fig. 3). The [Cys⁰] isomer exhibited faster and more extensive efflux.

Xenograft imaging and biodistribution studies

We utilized the INS-1 tumor as a GLP-1 receptor-positive tumor and MDA-MB-435 tumor as a negative control. Western blotting showed the relative amounts of GLP-1 in the two tumor types (Fig. 4). The PET images of FBEM-[Cys⁰]-exendin-4 clearly showed high uptake ($7.20 \pm 1.26\%$ ID/g at 1 h) in the INS-1 tumor and very low uptake in the MD-MB-435 tumor ($0.67 \pm 0.06\%$ ID/g at 1 h; Fig. 5a, b). The uptake in the INS-1 tumors was blocked by coinjection of 200 μ g/animal of [Cys⁰]-exendin-4 ($0.79 \pm 0.080\%$ ID/g at 1 h). The highest uptake was observed at the earliest time point, 30 min, and decreased thereafter (Fig. 5b). Quantification of the PET data at the 2-h time point showed acceptable tumor-to-kidney (0.74) and tumor-to-liver (2.49) ratios. Biodistribution studies using dissected tissues gave somewhat different tumor-to-kidney and tumor-to-liver ratios of 0.48 and 4.8, respectively (Fig. 5c).

However, FBEM-[Cys⁴⁰]-exendin showed even higher uptake in the INS-1 tumors based both on PET images ($25.25 \pm 3.39\%$ ID/g at 1 h) (Fig. 6a) and by biodistribution (Fig. 6b, c). Tumor uptake peaked at 1 h, while kidney uptake had already begun to clear by that time. The biodistribution determined by counting of dissected tissues following a 2-h uptake period showed excellent tumor-to-kidney (7.4) and tumor-to-liver (39.9) ratios. Analysis of PET ROIs showed a tumor-to-kidney ratio of 4.94 and a tumor-to-liver ratio of 10.1.

Specific binding in vivo was evaluated by i.v. injection of a blocking dose of [Cys^x]-exendin-4 10 min prior to administration of the corresponding [¹⁸F]FBEM-[Cys^x]-exendin-4 (Table 1). Significant blocking, both as a percentage and statistically, was observed in the INS-1 tumor, pancreas, stomach and lung for both exendin-4 isomers. With FBEM-[Cys⁴⁰]-exendin-4, the blocking dose resulted in a reduction in uptake in the intestine and increases in the kidneys, liver, and muscle.

Discussion

We have developed PET tracers for GLP-1R for potential imaging applications in neuroendocrine cancer, insulinoma, and diabetes. In addition, imaging applications may exist in cardiovascular disease because there is evidence that GLP-1R plays a role in cardiovascular function [24]. We wished to exploit the high resolution of PET by utilizing ¹⁸F, which has the lowest positron range of the commonly used PET isotopes. In addition, the quantitative ability of PET is better than that of SPECT. The development and validation of novel radiopharmaceuticals for PET require optimization of radiochemical reactions, product purification and characterization, and confirmation of the compound's suitability for its biological purpose.

We previously synthesized and studied a bis-cyclic amide peptide (EM3106B) that contained a C-terminal cysteine for radiolabeling with [¹⁸F]FBEM [18]. EM3106B was

designed to resist metabolism through inclusion of two internal lactam loops [19]. We observed high uptake into the INS-1 tumor xenograft model and appropriate blocking was observed in GLP-1R-containing tissues. Compared with the synthetic preparation of the bicyclic amide EM3106B, the linear peptide structure of exendin-4 provides easy C-terminal or N-terminal cysteine modification using standard solid-phase peptide synthesis methods. These cysteine-modified exendin-4 analogs would be amenable to radiolabeling using our [^{18}F]FBEM prosthetic group.

During the development of the chemical synthesis of these FBEM-[Cys^x]exendin-4 analogs, HPLC–MS analysis of the chemically synthesized peptides indicated the presence of an oxidation product that is easily separable by HPLC. The observation that the amount of the oxidation component varied inversely with the concentration injected onto the analytical HPLC column suggested that the analysis itself contributed to the oxidation. Inclusion of 1 mM EDTA in the mobile phase significantly reduced the amount of oxidation impurity observed during analytical HPLC.

One of the advantages of maleimide conjugation with a mono cysteine-containing peptide is the mild reaction conditions employed. The radiochemical reaction proceeds well at room temperature in PBS. We added 0.1% sodium ascorbate to the incubation medium to slow the formation of disulfide dimers, a technique we had utilized previously in the synthesis of Affibody-based radiochemicals [25]. Even with the inclusion of sodium ascorbate in the incubation medium for the radiochemical reaction, oxidized FBEM-[Cys^x]exendin-4 components were observed in varying amounts.

Previous studies on control of oxidative peptide modifications have indicated that methionine and tryptophan are the common sites of oxidation [26]. We made no attempt to determine the site of oxidation in our peptides. Concerns over the potential for methionine oxidation led to development of an exendin-4 analog named AC3174, which contains a leucine for methionine substitution [27]. Although the methionine oxidation was obviated, AC3174 has a shorter plasma half-life.

[^{18}F]FBEM-[Cys^x]exendin-4 that had been purified by HPLC was isolated from the eluate using C₁₈ solid-phase extraction and subsequent elution with 10 mM HCl in ethanol. HPLC analysis of this eluate showed a product of high radiochemical purity. However, after concentration of the solution under an argon stream, a significant amount of oxidation was found to have occurred. The flow of argon into an open tube can also draw air into the tube. Concentration using a rotary evaporator resulted in a significantly reduced amount of oxidation impurity.

The proteolytic stability of exendin-4 has previously been evaluated in human plasma [28]. The recovery of exendin-4 from human plasma using solid-phase techniques was about 50% and the half-life of the parent peptide was 9.57 h. The majority of the proteolytic degradation occurs in the N-terminal region. Since [^{18}F]FBEM-[Cys⁴⁰]exendin-4 displayed more favorable imaging results, we examined the in vitro stability of this isomer only. Because the half-life of ^{18}F limits the usefulness of these tracers to a few hours, we evaluated human plasma and serum stability for up to 1 h at 37°C for [^{18}F]FBEM-[Cys⁴⁰]exendin-4. Recovery of radioactivity from mouse serum following protein precipitation with acetonitrile was >80%, and the parent compound accounted for >88% of the extracted radioactivity. This percent recovery is much better than with the solid-phase method described above. Although the same high parent composition in extracted radioactivity was observed from human plasma, the extraction efficiency was only 72% and 68% at 30 and 60 min, respectively. This suggests that coprecipitation with plasma proteins may be a confounding factor for determining parent fraction in plasma.

For the purposes of biological validation of our new exendin-4 analogs, we selected the subcutaneous INS-1 tumor xenograft model. This is a convenient model, easily applied by a group with expertise in tumor xenograft models, to evaluate uptake of GLP-1R ligands. INS-1 expresses high levels of GLP-1R whereas MDA-MB-435, which we selected as a negative control, has very low expression (Fig. 4). Tissue uptake was evaluated utilizing static PET imaging and the obligatory tissue dissection biodistribution following the imaging studies. The biodistribution studies using tissue dissection are useful for measuring uptake in smaller tissues and tissues with low uptake.

Previous studies have demonstrated that modification of the N-terminal of exendin-4 decreases the biological activity as the N-terminal domain of exendin-4 is crucial for its agonist properties [4]. The N-terminal truncated exendin-(9-39)-amide is an antagonist at GLP-1R [29]. We demonstrated, using an in vitro binding assay, that the affinity of FBEM-[Cys⁴⁰]-exendin-4 was about twice that of FBEM-[Cys⁰]-exendin-4. In cell uptake studies, the uptake and internalization of C-terminal modified FBEM-[Cys⁴⁰]-exendin-4 was slightly higher than the N-terminal modified FBEM-[Cys⁰]-exendin-4. In addition the efflux of FBEM-[Cys⁰]-exendin-4 was somewhat faster.

Both isomers demonstrated highly selective INS-1 tumor uptake and both exhibited blocking of the uptake by coadministration of a large mass dose of unlabeled exendin-4 peptide (Figs. 5 and 6). Neither compound showed much uptake in the MDA-MB-435 control tumors. However, the uptake of C-terminal modified [¹⁸F]FBEM-[Cys⁴⁰]-exendin-4 was more than twofold higher in INS-1 tumors than that of the N-terminal modified [¹⁸F]FBEM-[Cys⁴⁰]-exendin-4. Although the differences noted between the two isomers in affinity, cellular uptake, and cellular efflux were consistent with this observation, the magnitude of the in vitro observations cannot explain the significantly higher uptake observed in vivo for [¹⁸F]FBEM-[Cys⁴⁰]-exendin-4. There may be differences in the metabolism due to the presence of the [¹⁸F]FBEM on the N-terminus of the [Cys⁰] isomer, but we have not yet studied this issue.

The peak tumor uptake with [¹⁸F]FBEM-[Cys⁰]-exendin-4 was at 30 min, whereas with [¹⁸F]FBEM-[Cys⁴⁰]-exendin-4 isomer the peak tumor uptake was at 1 h after injection. Uptake of [¹⁸F]FBEM-[Cys⁴⁰]-exendin-4 was 29%ID/g and 9.1%ID/g in INS-1 tumor and pancreas, respectively. Kidney uptake was high for both isomers, but because of the differences in tumor uptake, [¹⁸F]FBEM-[Cys⁴⁰]-exendin-4 had more favorable tumor-to-kidney ratios. Brom et al. [12] found INS-1 tumor uptake of 8.9%ID/g with [Lys⁴⁰(⁶⁸Ga-DOTA)]exendin-3 at 1 h and 25%ID/g with [Lys⁴⁰(¹¹¹In-DTPA)]exendin-3 at 1 h, which increased to 33.5% at 4 h. The pancreas uptake was 6.7%ID/g and 17.5%ID/g for the ⁶⁸Ga and ¹¹¹In tracers at the same time point, respectively. In our earlier work with [¹⁸F]FBEM-EM3106B, tumor uptake was 28.8%ID/g in INS-1 tumor and 5.45%ID/g in the pancreas. Thus [¹⁸F]FBEM-[Cys⁴⁰]-exendin-4 displays similarly high INS-1 tumor and pancreas uptake compared to these previously reported tracers.

Blocking studies were undertaken with both compounds to identify tissues that have saturable uptake. Both compounds displayed >87 % ($p<0.05$) blocking in the INS-1 tumor, pancreas, and lung. In addition, the [Cys⁴⁰] isomer showed 52% ($p<0.05$) inhibition in the intestine and increases of more than 49% in the liver, kidneys, and muscle. We have no explanation for these increases. Previous results of blocking studies in INS-1 xenograft models have also indicated high blocking in INS-1 tumor, pancreas, stomach, and lung [12]. INS-1 tumor, pancreas and lung are known to contain GLP-1R. These results suggest that both of these compounds specifically bind GLP-1R.

The pancreas would be the major organ of interest for applications of GLP-1R imaging agents for the study of diabetes and the detection of insulinomas. Since insulinomas typically occur in the pancreas, liver and intestinal uptake is the major background source to be evaluated. The results we obtained suggest the utility of [^{18}F]FBEM-[Cys⁴⁰]-exendin-4 as a PET imaging agent for GLP-1R-rich tissues. Based on PET quantitation, the [Cys⁴⁰] isomer was cleared from kidneys and liver resulting in a tumor-to-kidney ratio of 4.94 and a tumor-to-liver ratio of 10.1 at 2 h after injection. Although, we were unable to visualize the pancreas with our small-animal PET scanner, biodistribution calculated based on tissue dissection indicated a pancreas-to-liver ratio of 12.2. Thus, if all other parameters remain constant, imaging the pancreas and insulinoma in larger animals and humans should be possible.

Conclusion

The combination of high uptake in INS-1 tumor and pancreas and the ability to block this uptake with a high mass dose suggests that [^{18}F]FBEM-[Cys^x]-exendin-4 isomers have selectivity for GLP-1R. [^{18}F]FBEM-[Cys⁴⁰]-exendin-4 displays higher tumor uptake and better tumor to nontarget ratios than the [Cys⁰] isomer and, therefore, is the better candidate for further study as a GLP-1R-imaging agent.

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Fig. 1.
Competitive binding in INS-1 cells of $[^{125}\text{I}]\text{GLP-1}$ vs. various exendin-4 analogs

Fig. 2. Stability studies of [^{18}F]FBEM-[Cys 40]-exendin-4 in mouse serum and human plasma. Supernatants from protein precipitation were analyzed by radioHPLC. The radiochemical peak at about 18 min represents parent compound

Fig. 3. Cellular uptake, internalization, and efflux of [^{18}F]FBEM-[Cys 0]-exendin-4 and [^{18}F]FBEM-[Cys 40]-exendin-4 in INS-1 cells. **a, c** Uptake and internalization of (**a**) the [Cys 0] isomer and (**b**) the [Cys 40] isomer. Efflux of (**b**) the [Cys 0] isomer and (**d**) the [Cys 40] isomer

Fig. 4. Western blot of protein extracts of MDA-MB-435 and INS-1 tumor cells showing the relative levels of GLP-1R expression

Fig. 5.

a Representative PET images following injection of [^{18}F]FBEM-[Cys 0]-exendin-4 of mouse xenograft models of INS-1, INS-1 with a blocking dose of [Cys 0]-exendin-4 administered 10 min prior to the radiotracer, and MDA-MB-435. Animals were injected with 3.7 MBq radioactivity containing about 0.5–1 μg protein. The noses of the mice are to the top of the images. Each image is the best coronal plane to show the tumor uptake. **b** Time-course of uptake by selected tissues determined by quantitation of PET ROIs following injection of [^{18}F]FBEM-[Cys 0]-exendin-4 (3.7 MBq) into INS-1 and MDA-MB-435 xenograft models. **c** Biodistribution of [^{18}F]FBEM-[Cys 0]-exendin-4 at 2 h measured by radioactive counting of dissected tissues

Fig. 6.

a PET images following injection of [^{18}F]FBEM-[Cys 40]-exendin-4 of mouse xenograft models of INS-1, INS-1 with a blocking dose of [Cys 40]-exendin-4 administered 10 min prior to the radiotracer, and MDA-MB-435 control. Animals were injected with 3.7 MBq radioactivity containing about 0.5–1 μg protein. The noses of the mice are to the top of the images. Each image is the best coronal plane to show the tumor uptake. **b** Time-course of uptake by selected tissues determined by quantitation of PET ROIs following injection of [^{18}F] FBEM-[Cys 40]-exendin-4. **c** Biodistribution of [^{18}F]FBEM-[Cys 40]-exendin-4 at 2 h measured by radioactive counting of dissected tissues

Table 1

Inhibition of tissue uptake following coadministration of a blocking dose of 200 µg compared with uptake following injection of radioactive ligand alone. Tissue uptake was assessed by dissection and tissue counting. Negative values indicate increased uptake

Tissue	¹⁸ F]FBEM-[Cys ⁴⁰]		¹⁸ F]FBEM-[Cys ⁰]	
	Inhibition (%)	<i>p</i> -value	Inhibition (%)	<i>p</i> -value
Blood	9.06	0.338	−6.79	0.228
Spleen	49.00	0.284	35.13	0.322
Pancreas	97.96	1.37×10 ^{−4}	87.52	2.13×10 ^{−5}
Intestine	54.64	0.045	59.78	0.135
Stomach	91.50	0.005	88.58	0.021
Kidney	−99.15	0.005	44.16	0.066
Liver	−49.59	0.015	4.32	0.542
Heart	−27.85	0.261	89.78	0.417
Lung	91.12	3.83×10 ^{−4}	88.92	0.0035
Muscle	−166.97	0.007	38.64	0.766
Bone	−54.83	0.285	70.22	0.795
Tumor	98.67	3.43×10 ^{−5}	94.30	0.005